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SHORT REPORT

The promoter of the human cystic fibrosis transmembrane conductance regulator gene directing SV40 T antigen expression induces malignant proliferation of ependymal cells in transgenic mice

Frédéric Perraud¹, Kunihiro Yoshimura², Bernard Louis³, Wilfried Dalemans¹, Dalila Ali-Hadji¹, Huguette Schultz¹, Marie-Christine Claudepierre¹, Cécile Chartier¹, Claire Danel², Jean-Pierre Bellocq³, Ronald G. Crystal², Jean-Pierre Lecocq¹ & Andrea Pavirani¹

¹Department of Animal Systems, Transgene, 11 rue de Molsheim, 67082 Strasbourg Cédex, France; ²Pulmonary Branch, NHLBI, National Institutes of Health, Bethesda, Maryland 20892, USA; ³Service d'Anatomie Pathologique Générale, Hôpital de Hautepierre, 67200 Strasbourg, France

Transgenic mice bearing a human cystic fibrosis transmembrane conductance regulator (CFTR) promoter-SV40 T antigen fusion transgene were generated in order to localize *in vivo* the potential oncogenesis linked to the tissue-specific activity of the promoter for the CFTR gene. Surprisingly, the only site of tumors resulting from expression of the reporter *onc* gene was ependymal cells lining the brain ventricles. SV40 T antigen expression in these cells led to a consistent pathology in the first weeks of age: ependymoma and consequent hydrocephaly. Tumor-derived cell lines were established, characterized and shown to originate from SV40 T antigen-induced ependymoma. No pathological alterations were found in other organs, such as lungs and pancreas, in which cystic fibrosis is pathologically manifest in humans. Such transgenic mice and derived cell lines may represent valid models for analysing (1) the role of SV40 T antigen in ependymoma formation and (2) CFTR function in ependymal cells.

The major clinical manifestations of cystic fibrosis (CF), one of the most common autosomal hereditary diseases affecting Caucasians, involve the epithelial surface of lungs, pancreas and gastrointestinal tract (Boat *et al.*, 1989). As a result, normal respiratory and gastrointestinal functions are significantly altered. The clinical manifestations of CF are believed to result from abnormal electrolyte transport in the epithelial cells of these organs (Jetten *et al.*, 1989; Landry *et al.*, 1989; Levitan, 1989). CF is not a disease of, for instance, hematopoietic cells, mesenchymal cells, the endothelium or the central nervous system.

The disorder is caused by mutations in the gene coding for the cystic fibrosis transmembrane conductance regulator (CFTR) (Kerem *et al.*, 1989; 1990a,b; Riordan *et al.*, 1989; Rommens *et al.*, 1989; Cutting *et al.*, 1990; Dean *et al.*, 1990; Santis *et al.*, 1990; White *et*

al., 1990; Johansen *et al.*, 1991). There is increasing evidence that CFTR is a Cl⁻ channel, directly or indirectly modulating the epithelial transport of Cl⁻ in response to stimuli such as cAMP and protein kinases A and C (Anderson *et al.*, 1991a,b; Kartner *et al.*, 1991; Rich *et al.*, 1991; Wagner *et al.*, 1991). The normal and mutated products of the CFTR cDNA have been evaluated in heterologous systems, and complementation of the defective CF phenotype has been achieved *in vitro* by transfection of CF cells with normal CFTR cDNA (Cheng *et al.*, 1990; Drumm *et al.*, 1990; Gregory *et al.*, 1990; Rich *et al.*, 1990).

We have previously isolated and characterized the promoter of the human CFTR gene (Yoshimura *et al.*, 1991). Activity of the promoter has been demonstrated *in vitro* in epithelial cell lines, and the rate of transcription of the gene has been observed to be relatively low in normal human bronchial epithelium. In an effort to expand the understanding of the regulation and tissue-specific activity of the CFTR promoter, we have generated transgenic mice in which the CFTR promoter is linked to the SV40 T antigen, an *onc* gene that can induce tumors in the transgenic cells in which it is expressed (see, for instance, Hogan *et al.*, 1986; Palmiter & Brinster, 1986; Jaenisch, 1988; Babinet *et al.*, 1989; Hanahan, 1989). Further, the resulting tumor cells would be trans-immortalized, permitting establishment of permanent lines of cells with properties determined by the activity of the CFTR promoter (Pavirani *et al.*, 1989; Dalemans *et al.*, 1990; Jallat *et al.*, 1990; Perraud *et al.*, 1991).

Based on the clinical manifestations of CF, we expected that such an approach would permit the development of lung, pancreas and gastrointestinal epithelial tumors. Surprisingly, using a human CFTR promoter sequence fused to the SV40 early coding region as a reporter gene, the only tumors observed were ependymomas.

Transgenic mice develop a typical pathology: bulged cranium and hydrocephaly

A promoter fragment of the human CFTR gene containing 2244 bp upstream of the major transcription initiation site and 24 bp of 5' untranslated region (Yoshimura *et al.*, 1991) was placed in front of the

genomic SV40 early DNA region coding for the small t and large T antigens, nucleotides 5190–2533 (Tooze, 1981). This promoter region has been characterized previously (Yoshimura *et al.*, 1991), and has been shown to be transcriptionally regulatable (Trapnell *et al.*, 1991; Yoshimura *et al.*, 1991) and able to direct the *in vitro* expression of a reporter gene at low levels in cells of epithelial origin (Yoshimura *et al.*, 1991).

Using such a transgene, 23 founder transgenic mice were generated by pronuclear microinjection (Hogan *et al.*, 1986) of C57Bl/6 × SJL hybrid zygotes. These corresponded to 14.8% of the mice forming the founder litter. Such a percentage of viable transgenic pups is in the expected normal range, thus excluding fetal lethality as a result of transgene expression. We were able to examine 19/23 founders (four mice died overnight with consequent tissue autolysis or were cannibalized). A typical morphological abnormality of the head (bulged cranium) was evident in a total of 14/19 founders. This observation is consistent with a dominant rather than a random position integration effect of the transgene in the murine genome. Mice were sacrificed when terminally ill (average life of 4.5 weeks with a range of 1–8 weeks). Examination of their heads revealed a soft skull, the presence of intracerebral fluid and hydrocephaly.

Histopathology: ependymoma

Histopathological examination of 10 organs, including lungs, pancreas, intestine, liver, heart, stomach, spleen, kidney, genitals and thymus, from nine examined founder CFTR promoter-SV40 T antigen mice showed no abnormal pathology and preservation of normal tissue architecture in all specimens, except one mouse with a cardiac tumor. In contrast, in 8/9 founder animals examined, an obstructive hydrocephalus had dilated the ventricles and damaged the cortex to a considerable extent (Figure 1a). Tumor masses filled the four ventricles. Microscopic examination showed that the subependymal spaces were infiltrated by a primitive neuroepithelial tumor (Becker & Hinton, 1983) composed of a dense proliferation of small, primitive, malignant cells with round chromophilic nuclei and high mitotic activity (Figure 1a, arrow 1; Figure 1b). In some areas, anaplasia was pronounced, with multinucleated cells and a few cells with giant nuclei (Figure 1b). Within the ventricles, choroid papillary formations (Figure 1a, arrow 2) were composed of a single layer of cuboidal and some mucus-secreting columnar cells (positive with periodic acid-Schiff staining) on a thin vascular connective tissue stroma (Figure 1c and d). In several areas, the cells showed unequivocal malignant features, with multiple layers, marked pleomorphism, large hyperchromatic nuclei and high mitotic activity. In some areas, tumor nodules had developed from papillary plexus (Figure 1a, arrow 3), presenting either as tubular structures or as poorly differentiated cells with typical ependymal rosettes with a small lumen surrounded by a ring of ciliated cells (Figure 1e and f). Blepharoplasts could be seen in the cytoplasm of cells from both papillary and ependymal tumors (Figure 1d).

In summary, the lesions induced by CFTR promoter-SV40 T antigen fusion transgene were characterized as primitive neuroectodermal tumors that differentiated

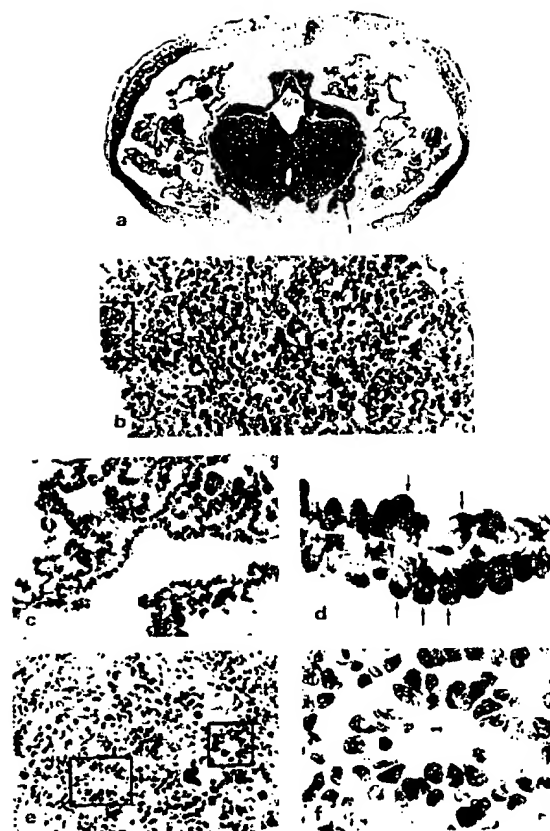


Figure 1 Pathology of the brain in transgenic mice expressing the human CFTR promoter-SV40 T antigen fusion transgene. (a) Longitudinal frontal section of the brain (mouse 12) showing the dilated ventricles as a result of hydrocephaly. The tumor is polymorphic with cells occupying the ventricle cavity presenting three different patterns of architecture (see numbered arrows): (1) a dense cellular mass infiltrating the subependymal spaces; (2) tumor cells arranged in papillary array; and (3) nodular structures within the ventricle. Hematoxylin-eosin stain. Magnification: $\times 4$. (b-f) Detailed images of the different tumor organizations (from mice 11 and 12). (b) Primitive neuroepithelial tumor (region indicated by arrow 1, a) composed of a sheet of pleomorphic undifferentiated cells with hyperchromatic nuclei and scant cytoplasm. Note the giant nuclei within the square (left). Specimen taken from mouse 12. Hematoxylin-eosin stain. Magnification: $\times 128$. (c) Papillary architecture (region indicated by arrow 2, a) composed of vascular connective tissue stroma and cuboidal or columnar cells with hyperchromatic nuclei. Specimen taken from mouse 12. Hematoxylin-eosin stain. Magnification: $\times 128$. (d) Blepharoplasts (arrows) in the apical cytoplasm of cells lining a papilla from c. These structures correspond to the basal bodies of ependymal cells. Specimen taken from mouse 12. Mallory's phosphotungstic acid hematoxylin stain. Magnification: $\times 400$. (e) Cellular malignant ependymoma with marked pleomorphism, giant cells undergoing mitosis forming the nodules (region indicated by arrow 3, a). Two typical ependymal rosettes are present within squares. Specimen taken from mouse 11. Hematoxylin-eosin stain. Magnification: $\times 128$. (f) Details of an ependymal rosette shown in e with multiple layers of ciliated cells surrounding a central lumen. Specimen taken from mouse 11. Hematoxylin-eosin stain. Magnification: $\times 400$.

into malignant ependymomas and choroid plexus carcinomas.

Correlation between SV40 T antigen expression induced by the CFTR promoter and the brain pathology

Immunohistochemical analysis performed on brain tissue of four CFTR promoter-SV40 T antigen trans-

genic founders using anti-SV40 T antigen monoclonal antibodies demonstrated a positive signal only in tumor tissue (Figure 2a). Fluorescence was manifest in proliferating malignant ependymal cells lining the floor of the ventricle, while interventricular papillary or nodular structures were negative. Cells from lungs, thymus, kidney and pancreas of the same transgenic mice were negative for SV40 T antigen fluorescence.

Trans-immortalized cells originate from ependymoma

Cells derived from 22 malignant ependymomas could be subcultured twice a week. They maintained a typical epithelioid morphology and had a doubling time of 17 h (Figure 3a). Cells were positive for the presence of SV40 T antigen mRNA (not shown) and for the presence of the SV40 T antigen protein in their nuclei as revealed by immunofluorescence analysis performed at

the fourth passage and later (Figure 2b). Characterization by electron microscopy after 10 passages and 25 generations demonstrated a polyhedral shape (Figure 3b and c). Several microvilli were present on the sur-

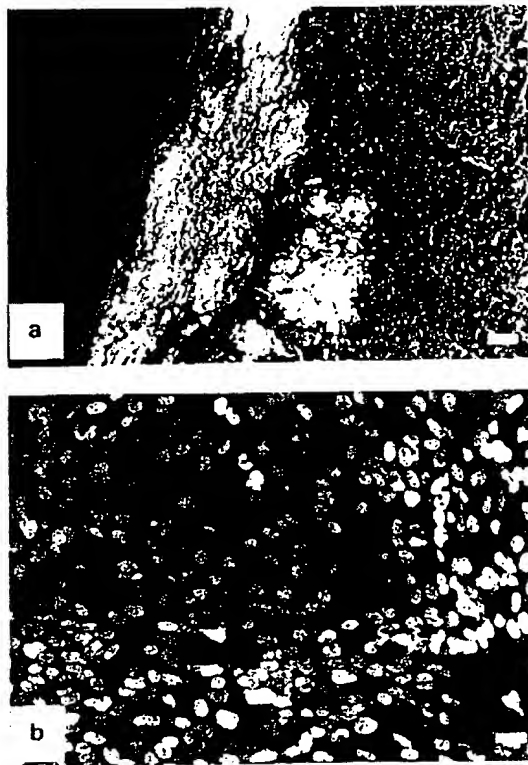


Figure 2 SV40 T antigen protein assessed by immunofluorescence in transgenic mice expressing the human CFTR promoter SV40 T antigen fusion transgene. (a) Longitudinal section of the brain of mouse 12 at 4 weeks of age revealing immunohistochemical detection of the SV40 T antigen in the ependymal cells lining the cavity of the ventricle (from region indicated by arrow 1 in Figure 1a). Bar: 500 µm. Paraffin-embedded tissue section slides were incubated overnight with anti-SV40 T antigen mouse monoclonal antibodies (PAB-419) (Harlow *et al.*, 1981), rinsed and rabbit anti-mouse IgG FITC-conjugated antibodies (ICN Immunobiologicals, Lisle, IL, USA) were subsequently applied for 2 h. (b) Nuclear localization of the SV40 T antigen in trans-immortalized cells derived from the brain tumor of mouse 7. Analysis was performed at the fourth passage in culture and revealed a homogeneous cell population positive for SV40 T antigen expression. Bar: 50 µm. Cells were cultured in Lab-Tek chamber slides (Nunc, Naperville, IL, USA) for 2 days, fixed with methanol: acetone (1:1) for 10 min and analysed with the antibodies described above, with the exception that incubation time was 1 h. Slides were mounted with a solution of N-propyl galate: ethanol (3:1) and examined by epifluorescent microscopy.

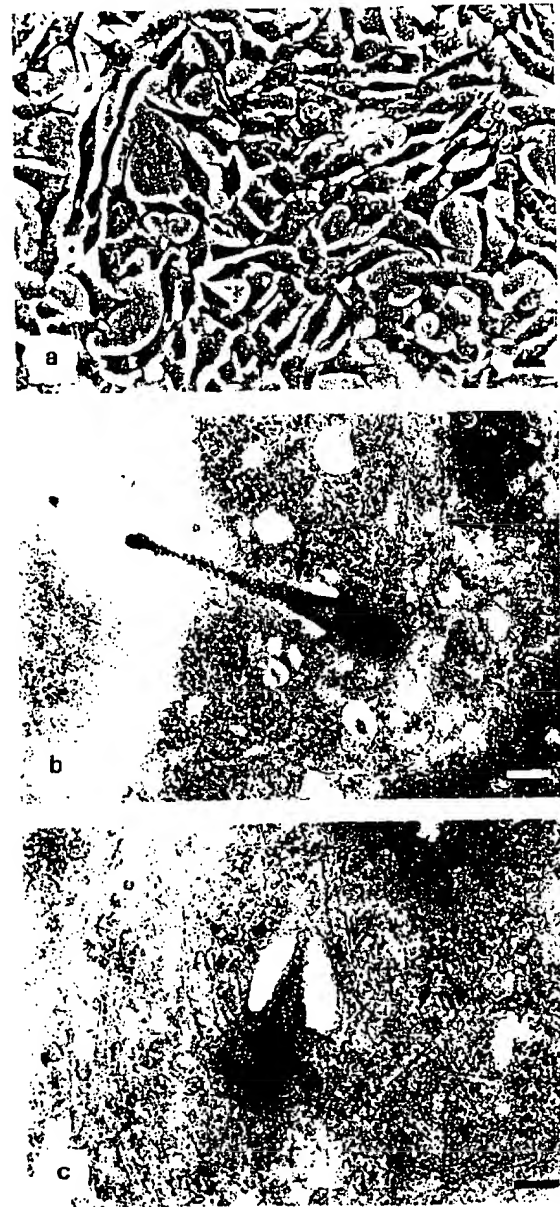


Figure 3 Morphology and ultrastructure of trans-immortalized cells derived from a brain tumor in transgenic mice expressing the human CFTR promoter SV40 T antigen fusion transgene. Cells were derived from mouse 7. Analysis was performed after ten passages in culture. (a) Phase contrast micrograph of cells. Bar: 50 µm. (b) and (c) Transmission electron microscopy with sections perpendicular to the monolayer surface. Cilia (c), basal bodies (bb), intermediate filaments (f) and microtubules (mt) typical of ependymal cells are visible. Bar: 0.5 µm. Tumor tissue was collected and cells dissociated by passage through a 1 mm diameter needle in a small volume of nutrient medium containing Waymouth MD705 basal medium (Gibco BRL, Cergy-Pontoise, France) supplemented with 10% fetal calf serum (FCS) (Gibco BRL, Cergy-Pontoise, France) and 40 µg ml⁻¹ gentamycin (Unicel, Lyon, France). After four passages, the initial complete medium was replaced by DMEM basal medium containing 10% FCS and 10 µg ml⁻¹ insulin (Sigma, St. Louis, MO, USA). Transmission electron microscopy was performed according to Perraud *et al.* (1991).

face, as well as complete cilia, consistent with an ependymal origin (Araki *et al.*, 1983; Gabrion *et al.*, 1988). The cytoplasm contained microtubules and few intermediate filaments in a random orientation (Figure 3b and c). No gap junctions and zonulae adherens were detected. As further evidence for their ependymal origin, the cells were positive for glial fibrillary acidic protein (Roessmann *et al.*, 1980) as demonstrated by immunofluorescence (not shown). In contrast, attempts to establish cell lines from histopathologically normal lung tissue of two CFTR promoter-SV40 T antigen transgenic mice were not successful.

In summary, the evidence presented in our work has a number of implications. Two observations suggest that the ependymal cell tumors resulted from the oncogenic potential of the human CFTR-driven SV40 T antigen, being manifest in the early development of the transgenic mouse brain. First, the brain damage was very severe, with the shape of the skull and the changes in normal brain tissue probably resulting from hydrocephaly ongoing for some time before birth. Second, the pathological analysis of the tumors suggested a primitive neuroectodermal tumor involving a primitive fetal neuroepithelial cell. Together, these findings imply that the human CFTR promoter is functional in the early development stage of the mouse and, possibly, that function of the CFTR product might be required early during the development of the brain. Unfortunately, the tissue-specific distribution of activity of the endogenous CFTR promoter during the development of the mouse is at present not known.

The fact that we failed to detect SV40 T-antigen protein in tissue such as lungs and pancreas does not prove that the human CFTR promoter fragment employed is not functional in these murine organs. It is possible that (1) ependymal cells are more prone to SV40 T-antigen-related tumorigenesis; (2) novel tissue specificity of the promoter may result from the structure of the chimeric transgene; (3) the human CFTR promoter may be more active in this murine cell type than in other murine cells; (4) the promoter fragment

employed lacks the regulatory DNA sequence necessary for specific expression in such mouse organs. Although choroid plexus tumors of ependymal origin generated by microinjection of a transgene carrying the SV40 promoter/enhancer and the SV40 T antigen as a reporter gene have been reported in transgenic mice (Brinster *et al.*, 1984; Messing *et al.*, 1985; Palmiter *et al.*, 1985), microinjection of a construct containing the SV40 promoter devoid of the 72 and 21 repeats evoked no significant pathology in transgenic mice (Palmiter *et al.*, 1985). Further, the SV40 T-antigen reporter used in the present study (which does not contain elements of the SV40 promoter/enhancer) has been used to target specific expression in lymphocytes (with the immunoglobulin heavy-chain enhancer/promoter) (Pavirani *et al.*, 1989), hepatocytes (with α_1 -antitrypsin promoter) (Perraud *et al.*, 1991) and lung epithelial cells (with the lung surfactant protein C promoter) (unpublished) of transgenic mice. In no case was hydrocephaly, brain tumor formation or T-antigen expression in brain observed in these animals.

Ependymal cells are of glial origin (Bruni *et al.*, 1985) and they are involved in ion and protein exchange and transport (Manthorpe *et al.*, 1977). This might logically suggest a functional role of the CFTR protein in those cells. If so, it is conceivable that sporadic mutations of the promoter of the CFTR gene might be relevant to some cases of central nervous system abnormalities in humans.

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